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A Study of the Effect of Isolation and Crystallization Procedures on the Electrophoretic Mobility of beta-Lactoglobulin by Use of a Novel Paper Strip Electrophoresis Apparatus.

by

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Inherent in all protein isolation procedures is the supposition that the end product obtained has an exact counterpart in the "native state" of the system from which it was separated (1). With the increasing refinement of analytical techniques, many "pure" crystalline proteins have been shown to be heterogeneous in respect to either their chemical or physical properties, or both.(2, 3, 4, 5). One possible source of the observed microheterogeneity in crystalline protein preparations has been ascribed to the effects of the purification techniques employed in their isolation (6, 7). These facts tend to cast doubt upon the initial supposition that the crystalline proteins have exact counterparts in the systems from which they were isolated. Comparison of the physical and chemical properties of proteins before and after isolation is difficult or impossible in most cases. However, some work has been done in this field.

Studies of the oxygen dissociation curves (8) and the spectral adsorption (9) of haemoglobin in the red blood cell and in systems containing isolated haemoglobin have indicated that this protein in its native environment has properties different from those observed after isolation has been affected.

During a study of the protein interactions that occur in milk

during the processing and storage of milk products, methods were developed for investigating the electrophoretic mobilities of the milk proteins in their native environment. A comparison of the electrophoretic mobilities of a sample of crystalline beta-lactoglobulin and the native whey proteins of milk was made in protein-free whey. This paper reports results obtained during this study which indicate that the isolation and crystallization techniques now commonly employed to produce "pure" beta-lactoglobulin do not affect the charge distribution on the surface of the molecules of this protein. Crystalline beta-lactoglobulin is shown to have an electrophoretically similar counterpart in the "native state" of the system from which it was isolated.

Since the electrophoretic apparatus used in this study is novel, its design and operational characteristics are described in some detail.

Equipment and Materials

A). Paper Strip Electrophoresis Apparatus: The apparatus used in this investigation was a variation of the simple design of Kunkel and Tiselius (10) in which an electrical potential is applied through large open electrode vessels to the ends of a buffer-dempened paper strip sandwiched between two pieces of plate glass. This design was modified to eliminate the bulky electrode compartments and minimize the amount of electrolyte used for an analysis. The electrical potential was applied to the paper strip through reversible silver-silver chloride electrodes embedded in blocks of agar containing KCl.

The apparatus was constructed from two blocks of Lucite[1/] each 24" x 4". One block was 3/4" thick, the other 1/2" thick. Two rectangular electrode compartments 4-1/8" x 2-1/8" x 1/2" were milled out of the ends of the thickest block. The distance between the inside edges of the compartments was 17-3/8". Electrodes consisting of four 3/8" O. D. coils of 1/16" silver wire were placed in each compartment and connected through a 1/16" hole drilled through the side of each well to terminals mounted on the side of the block. After the electrodes were electrolytically plated with silver chloride, the wells were filled to the top with a hot 2% agar solution containing 1% KCl. On cooling, the agar solidified around the silver-silver chloride coils to form a stable reversible electrode system.

In order to maintain the apparatus at constant temperature during operation, a water jacket 23" x 5" x 1/4" was cemented onto the outside face of each block.

The apparatus was held together by six 2" C clamps.

Direct current was obtained from a Spinco Duostat[1/] power pack.

B). Protein-Free Whey: A solution having very nearly the same composition and ionic activity as the phase in which the milk proteins are found in their native state, was prepared by dialyzing 200 ml. of distilled water against 40 liters of skim milk. The dialysis was carried on for 5 days with intermittent agitation at 5°C. Ten ml. of toluene were added to the milk before dialysis to prevent bacterial growth.

1/ The mention of trade names in this paper is for identification and implies no endorsement of the products.

C). Native Whey Protein Concentrate: Skim milk was centrifuged in the A Rotor of a model E Spinco[1] Ultracentrifuge for 60 minutes at 57,000 x G and 23°C. in order to remove colloidal caseinates. The supernatant solution was further centrifuged in the same rotor for an additional 6 hours at 150,000 x G and 23°C in order to concentrate the whey proteins. After centrifugation, all material except the pellet and the bottom 1 ml. of solution was removed from the tubes. The pellets were broken up into the residual solution in the tubes and allowed to stand at room temperature for approximately 1/2 hour. The material was collected and centrifuged in a clinical centrifuge for 15 minutes. The clear supernatant solution represented a concentration of whey proteins in unmodified whey. This solution was held under refrigeration in an atmosphere of toluene for electrophoretic analysis. These solutions were found to be relatively unstable, and were not stored for periods in excess of 3 days.

D). Purified beta-Lactoglobulin: Crystalline beta-lactoglobulin was prepared according to the method of Gordon and Ziegler (11). It was recrystallized three times. The crystals were collected by centrifugation and lyophilized. The dried material was stored under refrigeration. Subsequent analysis showed this preparation to be electrophoretically homogeneous in phosphate buffer at pH 7.5.

Experimental Methods

The electrophoretic characteristics of the proteins used in this study were determined on strips of Whatman #1 filter paper 22" long and ranging from 1/2" to 3" in width. The type of strip selected was wet with the solution in which the analysis was to be

carried out. Approximately 2.5 ml. of solution were required for each inch of strip width. Excess fluid in the strip was removed by blotting between sheets of filter paper. The damp strip was then placed on the surface of the electrode containing plastic block and the ends of the strip were brought into contact with the agar surfaces of the electrodes. Electrical contact between the agar and the paper strip was established by adding an additional drop of electrolyte to each end of the strip. A sample of the protein solution to be analyzed was placed on the paper near the center of the strip in the form of a spot or a line using either a micropipette or a Spinco[1/] applicator. Sample size used ranged from 1 lambda to 10 lambda depending on the protein concentration in the sample analyzed. Evaporation of water from the strip during electrophoresis was controlled by placing a Teflon[1/] gasket 1/4" wide and approximately .01 mm. thick around the outer edge of the block. The strip was then covered with the second Lucite[1/] block and the two blocks were clamped together using six 2" C clamps. An effort was made to obtain uniform pressure on the strip. The apparatus was equilibrated by allowing water of the desired temperature to circulate through the jackets for a 15 minute period prior to the start of the analysis. Water temperatures ranging from 10° to 23°C were used during the study. Direct current was then passed through the strip for periods of time adequate to achieve sufficient protein migration on the strip, the intervals ranging from 1 to 6 hours. On completion of the run the strip was removed from the apparatus, dried in a circulating air oven for 20 minutes at 130°C, and stained with bromphenol blue (10).

The electrodes were reversed in polarity after each run in order to maintain their silver chloride coating. If the buffers used in the analysis did not contain chloride, the electrodes were regenerated electrolytically after each week of operation.

The operational efficiency of the apparatus was determined by comparing strips obtained by its use with those obtained using a commercial model of the Durrum type apparatus (12). The fact that the new apparatus was water cooled enabled much higher potentials - up to 720 volts - to be placed across the strip. Therefore, equivalent migration and resolution could be obtained in much shorter time periods than were required by the use of the Durrum type apparatus.

An attempt was made to utilize gravitational forces to restrict electro-osmotic flow of water in the strip by studying the operation of the apparatus when the strip was held in positions other than horizontal.

The mobility of the crystalline beta-lactoglobulin dissolved back into whey was compared with the mobility of the whey proteins which had not been removed from their native environment. Whatman #1 paper strips were wet with protein-free whey, and samples of crystalline beta-lactoglobulin dissolved in whey and native whey protein concentrates were allowed to migrate through the strips under the influence of identical electrical potentials. The rate of migration of the two materials in the native environment of the milk proteins was compared.

In order to determine the effect of the presence of colloidal caseinates on beta-lactoglobulin in its native environment, small

amounts of the caseinates initially centrifuged out of the milk were added back to the native whey protein concentrate before electrophoretic analysis in protein-free whey.

Results

It was found that protein mixtures could be resolved and electrophoretic mobilities of proteins could be validly compared by use of paper electrophoresis apparatus equipped with reversible electrodes imbedded in a gelled electrolyte.

With this apparatus the electro-osmotic flow of water in the paper strips (13, 14) could be greatly reduced and in some cases could be completely eliminated. This was accomplished by operating the apparatus in a vertical or near vertical position to allow the force of gravity to act against the electro-osmotic forces operating in the paper strip.

The low electrolyte requirement of the apparatus made possible the determination of the mobility of some of the whey proteins in protein-free whey. Crystalline beta-lactoglobulin dissolved in protein-free whey was found to migrate through whey exactly as fast as the fastest component in the native whey protein concentrate, when both were subjected to fields of equal electrical potential.

Adding the caseinate complex back to the native whey protein concentrate caused a slight shift in the distribution of the fastest component towards the point of origin. The leading edge of the zone containing the fastest component maintained the rate of migration noted before the addition of the caseinate. The amount of fast-moving protein shifted toward the origin depended on the diameter

of the caseinate spot which remained at the point of application during the analysis. This possibly indicated that the beta-lactoglobulin might have been retarded by a protein-protein interaction with the caseinate as it migrated across the spot of application at the start of analysis.

Summary

1). A paper strip electrophoresis apparatus using low volumes of electrolyte was developed and tested.

2). With the use of this apparatus it was shown that crystalline beta-lactoglobulin has an electrophoretically similar counterpart among the whey proteins maintained in their native environment during the period of analysis.

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